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Differential effects of tumor necrosis factor-α and interleukin-1β on cell death in human articular chondrocytes

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Summary

Objective: The death of chondrocytes by apoptosis is characteristic of degenerative joint diseases, such as osteoarthritis (OA). Tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) have been shown to play an important role in the development of OA. In this study we analyzed the effects of TNF-α and IL-1β on cell death in normal human chondrocytes.

Methods: Normal human chondrocytes were isolated from knee cartilage obtained at autopsy from 30 adult cadaveric donors. The cells were stimulated with TNF-α (10 ng/ml) or IL-1β (5 ng/ml) in the presence or absence of Ro 31-8220 (Ro: a structurally related analog of bisindolylmaleimide that inhibits mitogen-activated protein kinase phosphatase 1 [MKP-1]) (Ro; 10 μM), an MKP-1 inhibitor, which induces apoptosis in chondrocytes. Apoptosis was evaluated by flow cytometry (propidium iodide) and nuclear morphology was evaluated with 4′,6′-dianidino-2-phenylindole dihydrochloride. The expressions of caspase-8, -7 and -3 and Bcl-2 were analyzed by Western blot and the activation of caspase-3 and -8 was measured by flow cytometry. Prostaglandin E2 (PGE2) was evaluated by enzyme-linked immunosorbent assay.

Results: At 24 h the percentage of apoptotic (hypodiploid) nuclei induced by TNF-α + Ro was higher than the level induced by Ro alone. The combination of IL-1β (5 ng/ml) with Ro did not show a synergistic effect. A morphological analysis demonstrated that treatment with TNF-α + Ro resulted in a large number of cells with condensed nuclei and DNA fragmentation. Western blot studies indicated that IL-1β + Ro did not induce the time-dependent activation of caspase-8, -7 and -3 as seen with TNF-α + Ro. As quantified by flow cytometry, TNF-α + Ro induced a higher level of caspase-3 and -8 activation than that seen with IL-1β + Ro. Pre-incubation for 2 h with caspase inhibitors for caspase-3, -7, -8 and pan-caspase significantly decreased the hypodiploid DNA peak induced by treatment with TNF-α + Ro at 24 h. Indomethacin increased the cell death induced by IL-1β + Ro; however, apoptosis induced by TNF-α + Ro was not modified by indomethacin.

Conclusions: These results confirm that TNF-α and IL-1β regulate apoptosis differently in this human chondrocyte model and that the differing effects of these cytokines are PGE2-independent. Indomethacin potentiates the effect of IL-1 on cell death and this may explain the reported effect of indomethacin on the progression of joint destruction.

Key words: Chondrocytes, Tumor necrosis factor-α, Interleukin-1β, Apoptosis, Caspase-3, Prostaglandin E2, Osteoarthritis.

Introduction

In most human articular diseases, chondrocyte death, and the resulting reduction of tissue cellularity, may be an important step in the pathogenesis of cartilage destruction[1,2]. The cytokines, interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), are particularly important in the pathophysiology of cartilage disease. Both *in vitro* and *in vivo* studies showed that the effects of TNF-α were similar to or synergistic with those of IL-1β[3]. However, the precise roles that TNF-α and IL-1β play in chondrocyte survival remain unknown[4]. Chondrocytes are normally resistant to the induction of apoptosis by IL-1β or TNF-α stimulation[5]. It has also been reported that IL-1β protects chondrocytes from CD95-induced apoptosis by a mechanism that is independent of IL-1β-induced nitric oxide (NO)[5]. However, conflicting results have been reported indicating that IL-1β-induced apoptosis in human articular chondrocytes[6]. The induction of chondrocyte apoptosis by TNF-α is uncertain because of differing results of several studies.

These results suggest that the pro- or anti-apoptotic effects of IL-1β or TNF-α may depend upon the experimental model. Differences in culture conditions (e.g., cell density, cell lines, monolayer or alginate beads) and the age of cartilage samples used for cell culture may contribute to this controversy. We recently described that TNF-α and IL-1β differently regulate activation of the apoptotic chondrocyte pathway in an *in vitro* model of apoptosis induced by actinomycin D (ActD) a DNA-dependent RNA polymerase inhibitor[7]. We demonstrated that this difference could be largely dependent on prostaglandin and caspase-8 levels. One significant aspect of this work is that the relationship between two key cytokines in the pathophysiology of articular disease and chondrocyte death was tested in the same experimental model and with the same culture conditions.

The current hypothesis to explain how cytokines can selectively kill some cells, while not being harmful to others, involves the operation of two opposite signaling pathways: a pre-existing destructive (apoptotic) pathway and an

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inducible protective (survival) pathway. Interactions between the two pathways, and particularly the balance of their activities, determine whether the cells survive or die[8]. Recently, significant progress has been made in clarifying the mechanism of apoptosis induced by cytokines, including TNF-α and IL-1. However, the identities of the protective factors induced by TNF-α or IL-1 and their mechanisms of action remain elusive. For example, TNF-α activation of extracellular signal-regulated protein kinase (ERK) and nuclear factor-κB (NF-κB) appears to counteract the cytotoxicity of the apoptotic pathway in certain cells[9,10], but not in others[11,12].

Some of the putative protective factors identified thus far include a manganese superoxide dismutase[13], a zinc finger protein[14], and members of the Bcl-2 family of proteins[15,16]. Mitogen-activated protein kinase (MAPK) phosphatase 1 (MKP-1) may act as a protective factor against apoptosis by suppressing the activation of c-Jun N-terminal kinase (JNK)[17]. MKP-1 down-regulation has been correlated to apoptosis induction in human chondrocytes[18]. Therefore, stimulation of cells with TNF-α + Ro 31-8220 (Ro: a structurally related analog of bisindolylmaleimide that inhibits MKP-1) may be useful as an *in vitro* model to study apoptosis. Given the complexity of the signaling pathways of cytokines, it is apparent that several protective factors may exert anti-apoptotic effects through a variety of mechanisms and may act at different stages of the apoptotic process in a manner dependent on cell type and stimulus.

In this study we activated the apoptotic pathways of human articular chondrocytes by culturing them with Ro. Our study demonstrates that TNF-α and IL-1β, in combination with an inhibitor of MKP-1, regulate the activation of the apoptotic pathway in human chondrocytes differently and that PGE2 is not a protective factor against the induction of apoptosis in human articular chondrocytes. These findings provide new clues for the understanding of cartilage degradation in articular disease.

Methods

CHONDROCYTE CULTURES

Normal human cartilage was obtained at autopsy from 30 adult cadaveric donors with no history of joint disease [inflammatory arthritis, osteoarthritis (OA), microcrystalline arthritis or osteonecrosis]. Under aseptic conditions, cartilage slices were removed from the condyles, minced and treated with trypsin (0.5 mg/ml) (Sigma–Aldrich, St. Louis, MO, USA) for 15 min at 37°C. After the cartilage was removed from the trypsin it was treated overnight with 2 mg/ml clostridial collagenase (Sigma–Aldrich) in Dulbecco's modified Eagle's medium (DMEM; Life Technology, Paisley, UK) in an orbital shaker at 37°C. The digest was centrifuged and the cells were resuspended in fetal calf serum (FCS)-enriched DMEM for culture in flasks. Subcultures were performed with trypsin-ethylenediaminetetraacetic acid (EDTA) (Gibco, Paisley, UK) and first passage cells were used. This study was approved by the Ethics Committee of Galicia – Spain.

CELL STIMULATION OF CHONDROCYTES

Chondrocytes were seeded into tissue culture plates (200 mm; Costar, Corning, NY, USA) for total protein extraction, six-well plates (Costar) for flow cytometric analysis and eight-well slides for morphological studies. Cells were rendered quiescent by 48 h of incubation in medium containing 0.5% FCS, washed and then stimulated with TNF-α (10 ng/ml) (R&D Systems, Europe) or IL-1β (5 ng/ml) (Sigma–Aldrich), in the presence or absence of Ro 31-8220 (Ro; 10 μM) (Sigma–Aldrich). Some cells were pre-incubated for 2 h with inhibitors of the caspases: caspase-3 inhibitor (Z-DEVD-FMK, 100 μM; BD Pharmmigen, Heidelberg, Germany), caspase-8 inhibitor (Z-IETD-FMK, 100 μM; BD); caspase-3 and caspase-7 inhibitor (5-[(S)-(−)-2-(Methoxymethyl)pyrrolidino]sulfonylisatin, 100 μM; Calbiochem, VWR International, Spain); general caspase inhibitor (Z-VAD-FMK, 100 μM; BD), a caspase-1 inhibitor (100 μM; BD); or an inhibitor of cyclooxygenase (COX) (indomethacin, Sigma–Aldrich). The cells were then stimulated with cytokines.

FLOW CYTOMETRIC ASSESSMENT OF CELL DEATH

Cellular DNA content was assessed by flow cytometry, as previously described[1]. For this purpose, the cells were cultured in six-well plates and cells attached to the plate were collected with trypsin and mixed with detached cells present in the supernatant. The cells were then centrifuged and resuspended in a solution containing 50 μg/ml propidium iodide (PI; Sigma–Aldrich), 1 μg/ml RNAse A (Sigma–Aldrich), 0.01% NP-40 (Sigma–Aldrich) in phosphate buffered saline (PBS). The cells were then incubated at 4°C for 30 min in the dark and analyzed by flow cytometry on FACScan (BD) using a 560 nm dichromatic mirror and 600 nm band pass filter. The percentage of cells with decreased DNA staining, which were apoptotic cells resulting from either fragmentation or decreased chromatin in a minimum of 20,000 cells per experimental condition, was determined. The data are expressed as the percentage of apoptotic (hypodiploid) nuclei. Cells with a very low DNA content, in which the type of cell death could not be ascertained, were excluded from the analysis. As assayed by flow cytometry, none of the solvents used for the different compounds at any dose used in our experimental conditions induced any significant degree of apoptosis.

MORPHOLOGICAL EVIDENCE OF APOPTOSIS

For morphological studies, chondrocytes were cultured in eight-well slides (Costar) and treated with Ro, Ro + IL-1β and Ro + TNF-α for 8 h. The cells were then washed with PBS, fixed in 10% formalin buffered saline for 10 min, stained with 4′,6-dianidino-2-phenylindole dihydrochloride (DAPI; 2 mg/ml, Sigma–Aldrich) for 30 min at 37°C, mounted in 90% glycerol/PBS, and observed by fluorescence microscopy.

WESTERN BLOT

After appropriate stimulation, cells were washed in ice-cold PBS, pH 7.5, and lysed in 0.2 M Tris–HCl, pH 6.8, containing 2% sodium dodecyl sulfate (SDS), 20% glycerol, 1 μg/ml cocktail inhibitor (Sigma–Aldrich) and 1 mM phenyl methyl sulfonyl fluoride (PMSF, Sigma–Aldrich). Whole cell lysates were boiled for 5 min and protein concentrations were determined using a bicinchoninic acid (BCA) reagent assay (Pierce Chemical Co., Rockford, IL, USA). Protein extracts (30 μg) were resolved on 12.5% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Immobilon P, Millipore Co., Bedford, MA, USA). Membranes were blocked in Tris-buffered saline, pH 7.4 containing 0.1% Tween-20, and 5% non fat dried milk for 60 min at room temperature, then incubated overnight with anti-caspase-8 (anti-human caspase-8, 1:700; MBL International, Woburn, MA, USA), anti-caspase-3 (mouse anti-human caspase-3, 1:2000), anti-caspase-7 (mouse anti-human caspase-7, 1:1000; BD, Madrid Spain), or anti-Bcl-2 (mouse anti-human Bcl-2; R&D, Abingdon, UK) at 4°C. After washing, the membranes were incubated with peroxidase-conjugated secondary antibodies and developed using an enhanced chemiluminescence (ECL) chemiluminescence kit (Amersham Biosciences/GE Healthcare, Barcelona, Spain). In order to assure that equal amounts of total proteins were loaded, we also hybridized each membrane with anti-tubuline (Sigma).

FLUORESCEIN ACTIVE CASPASE-3 ASSAY

The assay utilizes caspase-3 inhibitor (Z-DEVD-FMK) and caspase-8 inhibitor (Z-IETD-FMK) conjugated to fluorescein isothiocyanate (FITC) as the fluorescent *in situ* marker (BioVision Research Products, Mountain View, CA, USA). FITC-Z-DEVD-FMK and FITC-Z-IETD-FMK are cell permeable, non toxic and irreversibly bind to activated caspase-8 and -3 in apoptotic cells. The FITC label allows the direct detection of activated caspases in apoptotic cells by flow cytometry. After stimulation, the cells were washed in saline solution and incubated for 1 h with 1 μl FITC-Z-DEVD-FMK or FITC-Z-IETD-FMK at 37°C in 5% CO_2. A FACScan (Becton and Dickinson, Mountain View, CA, USA) with a fluorescein channel (FL1) detector, was used for the analysis.

QUANTIFICATION OF PGE2

The amount of prostaglandin E2 (PGE2) in the conditioned media was determined by a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Amersham Biosciences) according to the manufacturer's instructions. The sensitivity of the assay was 40 pg/ml, and the working range was between 50 and 6400 pg/ml.

STATISTICAL ANALYSES

The data are expressed as the mean ± standard error of the mean (S.E.M.) from determinations (*n*) or as representative results, as indicated. The statistical software program SPSS (version 12.0, SPSS, Chicago, IL, USA) was

used to perform analysis of variance (ANOVA) or Tukey tests. Differences were considered to be statistically significant at $P < 0.05$.

Results

IL-1β AND TNF-α DIFFERENTIALLY REGULATE CELL DEATH INDUCED BY Ro 31-8220 (Ro) IN HUMAN CHONDROCYTES

The stimulation of chondrocytes with the combination of TNF-α (10 ng/ml) and Ro (10 μM) significantly increased the hypodiploid peak induced by Ro (Ro: $7.99 \pm 5.3\%$; TNF-α + Ro: $23.05 \pm 7.3\%$, $n = 3$, $P < 0.0001$) at 24 h. In contrast, IL-1β (5 ng/ml) did not modify the effect of Ro in chondrocyte survival (IL-1β + Ro: $5.93 \pm 3.93\%$, $n = 3$, $P > 0.05$). Interestingly, IL-1β + Ro and TNF-α + Ro showed a significant difference in the percentage of hypodiploid cells ($P = <0.0001$) [Fig. 1(A)]. The morphological analysis of nuclei by immunofluorescence demonstrated that the treatment with TNF-α + Ro produced a large number of cells with condensed and fragmented nuclei; however, these morphologic changes in the nuclei were not observed with IL-1β + Ro treatment [Fig. 1(B)].

EFFECT OF IL-1β AND TNF-α ON CASPASE FAMILY PROTEINS IN HUMAN CHONDROCYTES

The expression of 35-kD and 33-kD unprocessed fragments of pro-caspase-7 and pro-caspase-3, respectively, was up-regulated in cultured chondrocyte cells after treatment with either TNF-α or IL-1β [Fig. 2(A)]. The combination of TNF-α + Ro decreased the level of 55-kD fragment of caspase-8, fragments of caspase-7 (35-kD) and caspase-3 (33-kD) were also clearly reduced in chondrocytes treated with TNF-α + Ro. However, IL-1β + Ro did not induce any modification in those fragments of pro-caspase-8, pro-caspase-7 or pro-caspase-3 [Fig. 2(A)]. Because reduction of pro-caspase fragments can not be interpreted as caspase activation we have carried out experiments showing that the inhibition of caspase-8 activity reverted

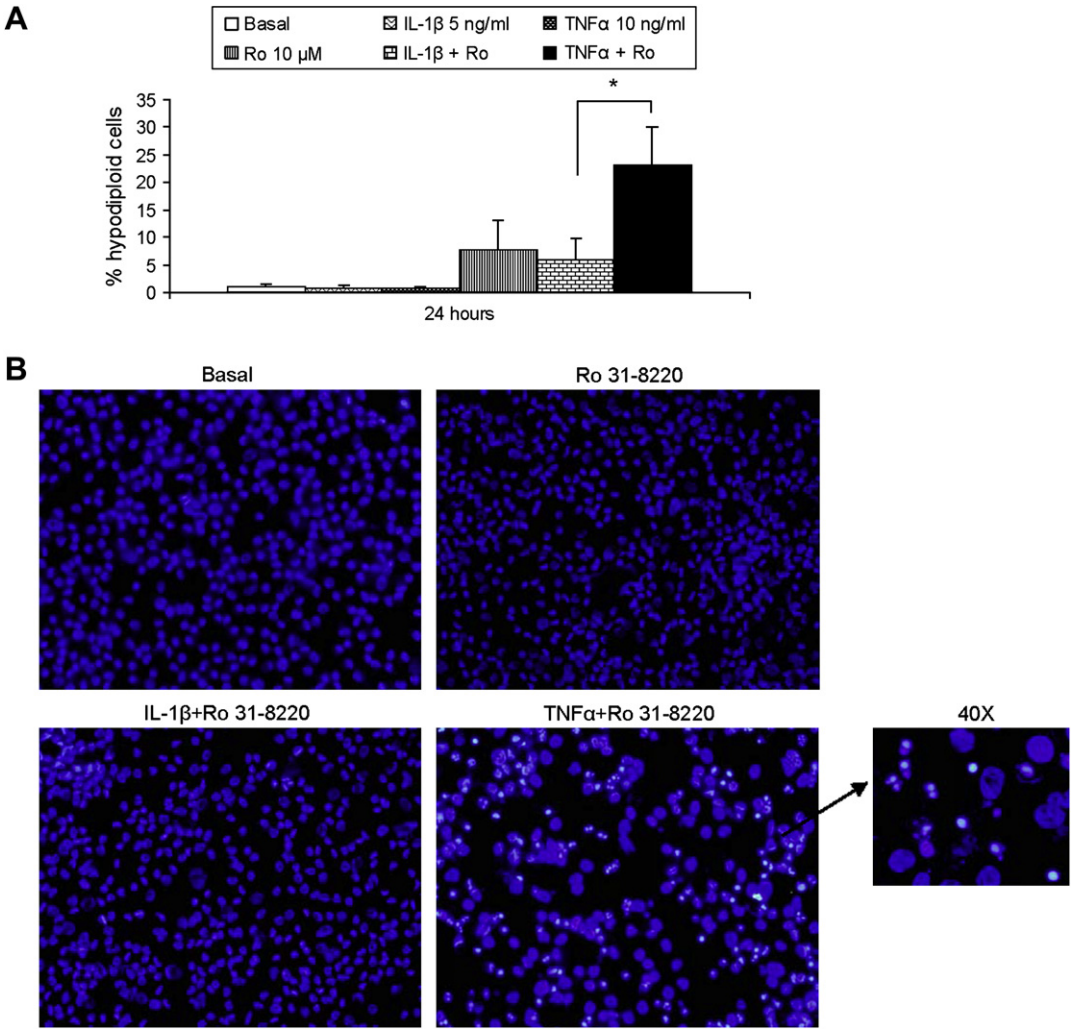

Fig. 1. Differential interaction of IL-1β and TNF-α with the cell death stimulator Ro 31-8220 (Ro). (A) Flow cytometry analyses of the DNA content in PI-stained cells were made in human chondrocytes that were incubated in six-well plates for 24 h with IL-1β (5 ng/ml), TNF-α (10 ng/ml), Ro (10 μM), or treated with IL-1β and Ro (5 ng/ml and 10 μM, respectively) or TNF-α and Ro (10 ng/ml and 10 μM, respectively). The data represent the mean ± S.E.M. of the percentage of cells with hypodiploid DNA content of three independent experiments in triplicate (*$P < 0.0001$ vs IL-1β + Ro). (B) Morphological studies using DAPI showed cellular changes characteristic of apoptosis in normal human chondrocytes treated with TNF-α and Ro for 8 h.

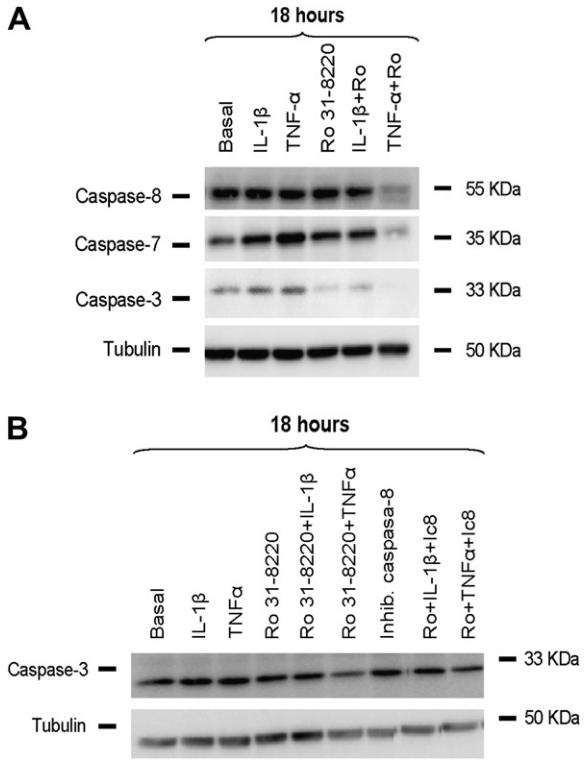

Fig. 2. Role of caspases family proteins in cell death difference between chondrocytes treated with IL-1β and TNF-α. (A) Aliquots of total cell lysates were first subjected to sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE); immunoblotting was performed using anti-caspase-8, -7 and -3 as described in Methods. Proteins were isolated from untreated cells, cells treated with IL-1β (5 ng/ml) or TNF-α (10 ng/ml), with or without Ro 31-8220 (Ro; 10 μM) for 18 h. Molecular size markers are shown on the right. Data are representative of three separate experiments. (B) Confluent chondrocytes were pre-incubated for 2 h with caspase-8 inhibitor and then stimulated with IL-1β or TNF-α or Ro 31-8220 (10 μM) or treated with IL-1β and Ro or TNF-α and Ro for 18 h. Aliquots of total cell lysates were subjected to SDS-PAGE; immunoblotting was performed using anti-caspase-3 antibody as described under Methods. Molecular size markers are shown on the left. Data are representative of three separate experiments.

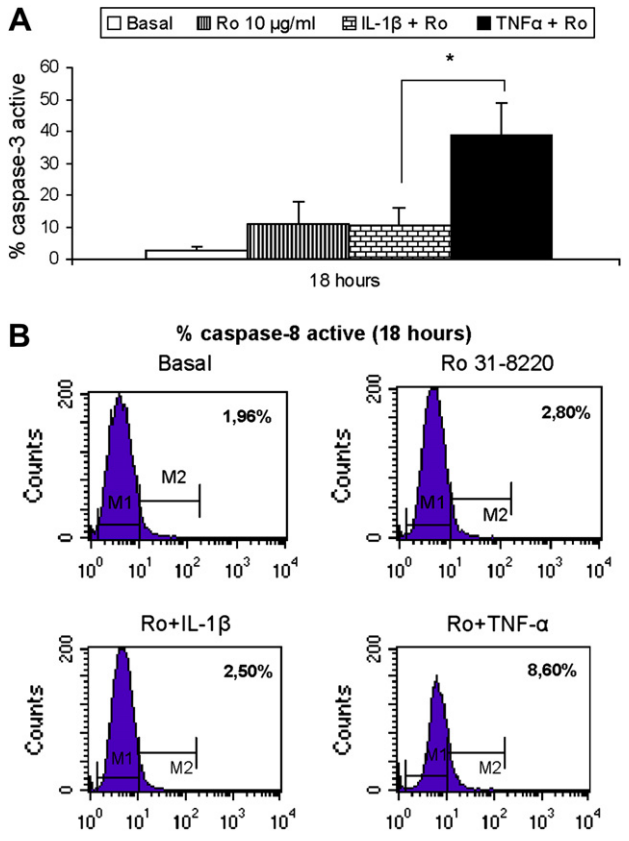

Fig. 3. Differential caspase activation in human chondrocytes treated with IL-1β and TNF-α (A and B). Flow cytometry quantification of active caspase-3 and -8 was made in human chondrocytes that were incubated in six-well plates for 18 h with IL-1β (5 ng/ml), TNF-α (10 ng/ml), Ro (10 μM), or treated with IL-1β and Ro (5 ng/ml and 10 μM, respectively) or TNF-α and Ro (10 ng/ml and 10 μM, respectively). The combination of TNF-α + Ro significantly increased the active fragments of caspase-3 (*$P < 0.01$) and -8 compared to IL-1β and Ro. Data represent the mean ± S.E.M. of the percentage of cells with active caspase-3 of three independent experiments in triplicate and data represent the percentage of cells with active caspase-8 of one experiment.

the reduction of pro-caspase-3 induced by TNF + Ro [Fig. 2(B)].

In order to confirm activation of the caspases, we also evaluated the active forms of caspase-3 and caspase-8 by flow cytometry. The results indicated that, at 18 h, Ro (10 μM) induced a slight increment in caspase-3 and caspase-8 activity compared to the basal level. The combination of TNF-α + Ro significantly increased the active fragments of caspase-3 and caspase-8 compared to Ro [Fig. 3(A) and (B)]. However, IL-1β + Ro did not activate caspase-3 and caspase-8 when compared to Ro [Fig. 3(A) and (B)].

ROLE OF BCL-2 AND THE CASPASES IN THE DIFFERENTIAL EFFECT OF IL-1β AND TNF-α

To elucidate the different effects of TNF-α and IL-1β in the apoptotic model using Ro, we first analyzed the role of Bcl-2, an anti-apoptotic protein. The results showed that both cytokines, TNF-α and IL-1β, induced Bcl-2 protein expression in human chondrocytes (Fig. 4). However, when Ro was present, neither TNF-α nor IL-1β modified the level of Bcl-2 (Fig. 4). These results suggest that Bcl-2 is not the cause of the differential effects of IL-1β and TNF-α in this *in vitro* model of cell death.

To elucidate the role of the caspases on the effect of TNF-α on chondrocyte survival, we employed inhibitors of caspase-3 (Ic3), caspase-3/7 (Ic3-7), caspase-8 (Ic8), and a pan-caspase inhibitor (Icgen). The results showed that pre-incubation for 2 h with all the caspase inhibitors significantly decreased the hypodiploid DNA peak induced by stimulation with Ro (10 μM) + TNF-α (10 ng/ml) for 24 h (TNF-α + Ro = 17.04 ± 1.15%; TNF-α + Ro + Ic3 = 2.86 ± 0.60%; TNF-α + Ro + Ic3-7 = 5.93 ± 3.45%; TNF-α + Ro + Ic8 = 3.46 ± 1.11%; TNF-α + Ro + Icgen = 2.96 ± 0.66%, $n = 3$, $P < 0.0001$) (Fig. 5).

IMPLICATION OF PGE2 IN THE DIFFERENTIAL EFFECTS OF IL-1β AND TNF-α

Prostaglandins participate in the pathogenesis of cartilage degradation. In human articular chondrocytes treatment with IL-β at 5 ng/ml for 24 h induced significantly

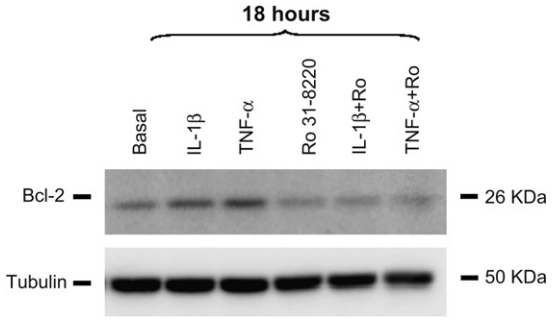

Fig. 4. The effects of treatment with IL-1β and TNF-α on Bcl-2 protein expression by human chondrocytes. Confluent chondrocytes were incubated for 18 h and proteins were isolated from untreated cells, cells treated with IL-1β (5 ng/ml) or TNF-α (10 ng/ml), with or Ro 31-8220 (Ro; 10 μM) for 18 h. Aliquots of total cell lysates were subjected to SDS-PAGE; immunoblotting was performed using anti-Bcl-2 antibody as described under Methods. Molecular size markers are shown on the right. The data are representative of three separate experiments.

higher levels of PGE2 than did TNF-α at 10 ng/ml (basal = 4.67; IL-1 = 304.1; TNF-α = 6.7 pg/ml, $P < 0.0001$) [Fig. 6(A)]. When Ro was present in the medium, PGE2-induction by IL-β was significantly reduced to near basal levels (11.77 pg/ml; $P < 0.0001$) [Fig. 6(A)]. PGE2-induction by TNF-α was not modified by Ro [Fig. 6(A)]. To investigate whether prostaglandins other than PGE2 contribute to the differing responses of IL-β and TNF-α to the apoptosis induced by Ro, we carried out experiments employing indomethacin. The results demonstrated that indomethacin at 100 μM increased the percentage of apoptotic chondrocytes induced by IL-β + Ro (IL-β + Ro = 8.43%; IL-β + Ro + indomethacin = 16.45%; $n = 3$, $P < 0.01$). However, apoptosis induced by TNF-α + Ro was not modified by indomethacin [Fig. 6(B)].

Discussion

In this report, we have demonstrated that both TNF-α and IL-1β, in combination with an inhibitor of MKP-1, differentially regulate the activation of the apopototic pathway in human chondrocyte cells.

We employed an *in vitro* model with Ro 31-8220 (Ro) to study the apoptosis of chondrocytes. Ro inhibits MKP-1, which is responsible for suppressing prolonged activation of JNK in some cells, thereby protecting them from apoptosis[18,19]. In this model Ro induced apoptosis in 10% of human chondrocytes. The combined treatment with TNF-α and Ro caused increased cell death in a dose- and time-dependent manner, that, interestingly, was not found using IL-1β with Ro (data not shown). In addition, we confirm that neither cytokine alone is capable of inducing cell death. Our results have demonstrated that the increase in the hypodiploid DNA peak and DNA fragmentation induced by Ro was amplified by TNF-α, but not by IL-1β. When the activity of caspase-3 and capase-8 was evaluated, we found that the IL-1β + Ro combination did not induce the increase in the activation of caspase-3 and caspase-8 that was observed by incubation with TNF-α + Ro.

To further define the molecular mechanisms of sensitization to IL-1β and TNF-α-induced apoptosis we analyzed three candidates: Bcl-2 protein, the caspases and prostaglandins, specifically PGE2. It has been reported that both TNF-α and IL-1β induce the anti-apoptotic family, Bcl-2, in chondrocytes[6,7,20]. In this work we demonstrated that Bcl-2 similarly decreased in the presence of IL-1β + Ro and TNF-α + Ro. Similar results were obtained when Mcl-1 (an anti-apoptotic protein) was studied (data not shown). This would seem to indicate that Bcl-2 and Mcl-1 are not responsible for the difference between the abilities of IL-1β and TNF-α to initiate pro-apoptotic signaling cascades.

TNF-α is known to induce apoptosis in diverse cells through the recruitment of caspase-3. It has been previously described that TNF-α/ActD-induced cell death occurred by apoptosis and that it was associated with caspase activation[7,21]. In this study, we investigated the relative contribution of the caspases to IL-1β- and TNF-α-induced cell death in the presence of Ro. The results showed that TNF-α, but not IL-1β, increases caspase-3 and -8 activities. Furthermore, cell death was significantly inhibited by a caspase-8 inhibitor and a caspase-3 inhibitor in TNF-α + Ro-treated chondrocytes, confirming that cell death induced by TNF-α + Ro is caspase-dependent. Although IL-1 increased the basal level of pro-caspases -3, -7 and -8, IL-1 did not modify the apoptosis induced by Ro. This finding is

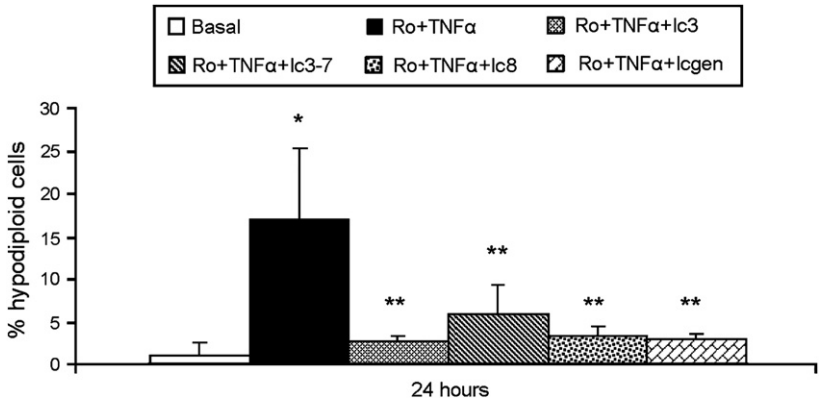

Fig. 5. Role of the caspases in the interaction of TNF-α with the cell death stimulator, Ro 31-8220 (Ro). Flow cytometry analyses of the DNA content of PI-stained chondrocytes pre-incubated for 2 h with inhibitors (100 μM) of caspase-3 (Ic3), caspase-3/7 (Ic3-7), caspase-8 (Ic8) or a pan-caspase inhibitor (Icgen); the cells were then stimulated with Ro (10 μM) + TNF-α (10 ng/ml) for 24 h. All the inhibitors of the caspases significantly decreased the hypodiploid DNA peak induced by stimulation with Ro + TNF-α (*$P < 0.0001$ vs basal untreated cells and caspase inhibitors and **$P < 0.0001$ vs Ro + TNF-α). Values are the means ± S.E.M. of three experiments performed in duplicate.

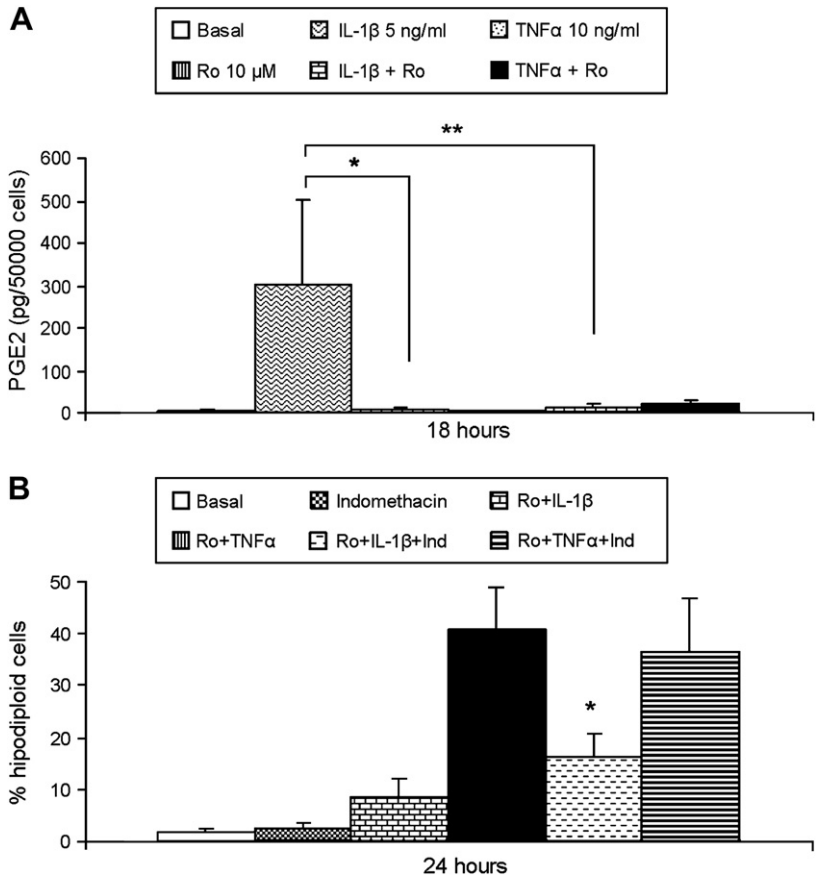

Fig. 6. Implication of PGE2 in the differential interaction of IL-1β and TNF-α with the cell death stimulator, Ro 31-8220 (Ro). (A) The amounts of PGE2 in the conditioned media of chondrocytes incubated in medium alone or treated with IL-1β (5 ng/ml) or TNF-α (10 ng/ml), with or without Ro (10 μM), were determined at 18 h by an enzyme-linked immunosorbent assay. PGE2 levels are expressed as the mean ± S.E.M. of four experiments performed in duplicate (IL-1β vs basal and TNF-α, $^{*}P < 0.0001$; IL-1β vs Ro + IL-1β, $^{**}P < 0.0001$). (B) Flow cytometric analysis of the DNA content of PI-stained chondrocytes pretreated with the COX inhibitor, indomethacin (100 μM), for 2 h, followed by co-incubation with IL-1β + Ro or TNF-α + Ro for an additional 24 h. Indomethacin pretreatment increased the percentage of apoptotic chondrocytes induced by IL-β + Ro ($^{*}P < 0.05$ vs Ro + IL-1β). The data represent the mean ± S.E.M. of the percentage of cells with hypodiploid DNA content of four independent experiments in duplicate.

supported by the results showing not modulation of any level of pro-caspase when IL-1 was combined with Ro. These results indicate possibly different requirements for the caspases during TNF-α + Ro or IL-1β + Ro interactions.

Few studies have used the same model to examine the differential effect of IL-1 and TNF on chondrocytes survival. Recently, a study investigating the interaction between TNF and IL-1 in an animal model was reported[22]. These authors suggest that TNF-mediated cartilage damage is completely dependent on IL-1. Our results are in concordance with the findings of this study, confirming that TNF plays an important role in chondrocyte death. We have not determined whether the final effect of TNF in chondrocyte death is IL-1 dependent. However, our results support the idea that IL-1 alone is not enough to induce death in chondrocytes. Perhaps IL-1 needs a co-factor induced by TNF or by indomethacin to induce apoptosis in chondrocytes.

Multiple reports have shown that PGE2 plays a crucial role in the pathogenesis of OA[23,24]. Increased COX-2 expression has been reported in cartilage and synovial tissues from patients with OA or rheumatoid arthritis (RA), and also in several models of cartilage degradation[23–25]. Some findings suggest that PGE2 mediates the IL-1β effect on cartilage degradation[26]; however, PGE2 may also have positive effects on cartilage by increasing the level of glucocorticoid receptors in chondrocyte cells, influencing cartilage differentiation and proliferation, and mediating the effects of vitamin D on cartilage[27]. Similar results were found in our previous studies using the actinomycin model and from another report that found that TNF-α-mediated protection of chondrocytes from NO-induced apoptosis requires COX-2 activity[28]. Whether PGE2 causes cell death is highly controversial[6,28–30]. In our study, we found that the levels of PGE2 induced by IL-1β were much higher than those induced by TNF-α. Interestingly, when Ro was present in the culture medium, the IL-1β-induced PGE2 levels were significantly reduced to near basal levels. This suggests that PGE2 levels do not play a role in the apoptosis induced in chondrocytes by IL-1 + Ro. We then examined, by the inhibition of endogenous prostaglandin synthesis, whether other molecules synthesized by COX could contribute to the different effects of IL-1β and TNF-α on cell death. We found that indomethacin resulted in an increase in cell death induced by IL-β in the presence of Ro [Fig. 5(B)]. These data suggest that the synthesis of

some molecules inhibited by indomethacin could explain the different effects of IL-1 and TNF on chondrocyte death. Similarly, it has recently been established that the mitochondrial protein, Smac/Diablo, is essential for the apoptosis induced by non-steroidal anti-inflammatory drugs (NSAIDs) in colon cancer cells[31]. It has also been reported that 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ2), a natural ligand of the peroxisome proliferator-activated receptor-γ (PPARγ), has an anti-apoptotic effect on chondrocytes and also synovial fibroblasts through inhibition of ERK1/2 phosphorylation[32].

The clinical relevance of the results of this study is focused on at least two therapeutic areas in OA treatment. One area is support for the idea that indomethacin (a classical NSAID) may in some circumstances, such as high levels of IL-1 and inhibition of the MKP-1 pathway, cause damage to articular cartilage. This may explain the accelerated destructive effect of indomethacin in hip OA described several years ago[33]. Another consideration from our results is that the blocking of cytokines such as TNF-α may prove clinically useful for prevention of cartilage degradation in OA.

In summary, this set of studies provides evidence that both TNF-α and IL-1β, in combination with an inhibitor of MKP-1, differentially regulate the activation of the apoptotic pathway in human chondrocyte cells. Furthermore, PGE2 does not modulate apoptosis in this *in vitro* model and indomethacin increases the apoptosis induced by IL-1 + Ro. These data are important for a better understanding of the participation of TNF-α and IL-1β in the pathogenesis of cartilage degradation and may provide new clues for understanding the treatment of OA with cytokine antagonists.

Conflict of interest

The authors have no conflict of interest.

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